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# Rasip1 is essential to blood vessel stability and angiogenic blood vessel growth

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## SUMMARY

Cardiovascular function depends on patent, continuous and stable blood vessel formation by endothelial cells (ECs). Blood vessel development initiates by vasculogenesis, as ECs coalesce into linear aggregates and organize to form central lumens that allow blood flow. Molecular mechanisms underlying *in vivo* vascular 'tubulogenesis' are only beginning to be unraveled. We previously showed that the GTPase-interacting protein called Rasip1 is required for the formation of continuous vascular lumens in the early embryo. Rasip1$^{-/-}$ ECs exhibit loss of proper cell polarity and cell shape, disrupted localization of EC-EC junctions and defects in adhesion of ECs to extracellular matrix (ECM). *In vitro* studies showed that Rasip1 depletion in cultured ECs blocked tubulogenesis. Whether Rasip1 is required in blood vessels after their initial formation remained unclear. Here, we show that Rasip1 is essential for vessel formation and maintenance in the embryo, but not in quiescent adult vessels. Rasip1 is also required for angiogenesis in three models of blood vessel growth: *in vitro* matrix invasion, retinal blood vessel growth and Directed *In vivo* Angiogenesis Assays (DIVAA). Rasip1 is thus necessary in growing embryonic blood vessels, postnatal angiogenic sprouting and remodeling, but is dispensable for maintenance of established blood vessels, making it a potential anti-angiogenic therapeutic target.

## Graphical abstract

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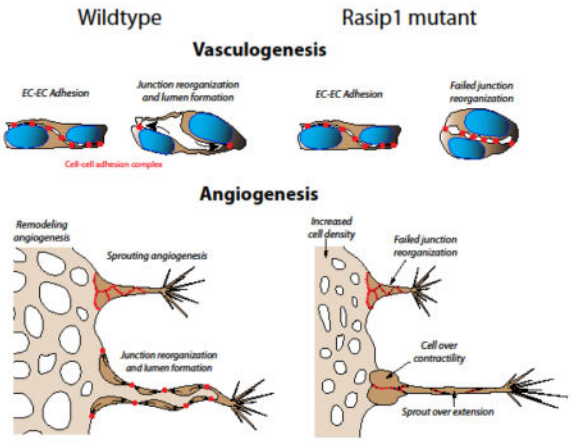
**COMPETING INTERESTS**

The authors declare no competing or financial interests.

**AUTHOR CONTRIBUTIONS**

Most experiments were performed by Y.K. K.X. initiated experiments by crossing mouse lines and making initial observations. D.M.B. and S.F. carried out supportive experiments and D.M.B. finished key studies. K.T. and C.M. assisted with DIVAA implants. G.E.D. contributed to underlying ideas and analysis, contributed 3D *in vitro* data, and read manuscript critically. O.C. supervised the overall project and contributed to the analysis. Y.K., D.M.B and O.C. wrote the manuscript.




**Keywords**

Rasip1; blood vessel; endothelial; lumen; tubulogenesis; angiogenesis; vasculogenesis; vascular; VE cadherin

## INTRODUCTION

Formation of a blood vessel network by both vasculogenesis and angiogenesis is essential for embryonic development, as well as for both normal and pathological tissue functions in the adult. Blood vessels are initially composed solely of endothelial cells (ECs), although they come to recruit supportive mural cells. During development, *de novo* vessel tubulogenesis termed ‘vasculogenesis’ occurs when EC progenitor cells, or angioblasts, assemble at embryonic day 8 (E8.0). Initially, they aggregate into linear cord structures, which then open a central cavity (or lumen). This event allows blood flow, providing oxygen and nutrition to surrounding tissues. Afterwards, new blood vessels sprout from pre-existing blood vessels via a process called ‘angiogenesis’. Growing blood vessels expand their lumens and remodel to form a vascular network and a functional circulatory system. The mechanisms underlying formation of functional lumens during blood vessel development are still not completely understood.

### Vascular tubulogenesis

While lumen formation has been extensively studied *in vitro* using endothelial [1–3] and epithelial systems [4], the question has recently attracted attention in mammalian *in vivo* models [5–7]. Human umbilical vein endothelial cells (HUVECs) in three dimensional (3D) collagen matrices have been used to identify molecules required for lumen formation, including members of the Rho family GTPases such as Cdc42 [2,8]. Since then, entire molecular cascades have been identified in this process [3]. More recently, mammalian vascular lumen formation was shown to fail in portions of the dorsal aortae of the classical mouse vascular endothelial growth factor A (VEGF-A) mutant [9]. In addition, the negatively charged sialomucin podocalyxin (PODXL) at the apical surface of mouse aortic ECs was shown to catalyze lumen initiation via electrostatic repulsion [10].

Cell adhesion, both between ECs and to surrounding extracellular matrix (ECM), is also essential to proper vessel tubulogenesis. Vascular endothelial cadherin (VE-cadherin or VEcad), a cell adhesion molecule, must be removed from the apical surface of ECs to allow lumen opening, however tubulogenesis is impaired in VEcad null mutants [9]. Similarly, loss of β1-integrin in embryonic ECs leads to loss of cell polarity and a block of lumen formation in arterioles [6]. Together these studies have advanced our understanding of vascular lumen formation, however additional molecular regulators of blood vessel formation continue to be discovered.

## Rasip1 and GTPases - roles during lumen formation

We showed that Ras interacting protein 1 (Rasip1) is essential for embryonic blood vessel morphogenesis [7]. Rasip1 was initially identified in a yeast two-hybrid screen that identified binding partners of Ras [11]. We showed that Rasip1 was an EC-specific factor throughout embryonic development and into post-natal stages [12]. Deletion of Rasip1 in both frog and mouse caused vascular failure [13]. Rasip1 null ECs exhibit disrupted polarity of junctional complexes, as well as loss of adhesion to ECM. Rasip1 depletion in early blood vessels resulted in discontinuous vascular tubes, blocking blood flow and leading to embryonic lethality by E10.0. Depletion of Rasip1 in ECs cultured in 3D matrices similarly led to failed tubulogenesis. Furthermore, Rasip1 was found to positively regulate the activity of Cdc42 and Rac1, while inhibiting RhoA via the GTPase activating protein (GAP) Arhgap29 during endothelial tubulogenesis. A key role of Rasip1 was shown in control of RhoA signaling and cytoskeleton contractility, which impacted both cell adhesion and EC cell shape, and thereby morphogenesis of blood vessels.

Other groups have identified Rasip1 as a Rap1 effector that controls cell adhesion and junctional stabilization [14]. Rasip1 was shown to signal via Rap1 and its GEF Epac1 and via non-muscle myosin IIB to control cell-cell interactions. This study suggested that Rasip1 primarily regulates the integrity of EC-EC adhesions and blood vessel permeability. More recent studies have confirmed aspects of Rasip1 signaling, placing it upstream of Rap1 and cell-cell junctions, but also identifying it as a key regulator of RhoA signaling via Arghap29 in control of the cytoskeleton and cell adhesion [15,16]. Together, these findings underscore the critical importance of Rasip1 for normal morphogenesis of blood vessels and open questions as to how it regulates formation of vessels across diverse vascular beds.

Here, we show that in addition to its early role in vasculogenesis, Rasip1 is essential for later vessel development, including fetal vascular lumen maintenance, as well as both fetal and postnatal vessel sprouting and remodeling angiogenesis. Using a newly generated Rasip1 conditional allele and a variety of ubiquitous or inducible endothelial Cre driver lines, we deleted Rasip1 at different developmental time points, as well as in quiescent adult vessels. Notably, deletion of Rasip1 from the onset of embryogenesis using Sox2-Cre phenocopied our original global Rasip1 deletion model [7]. We find that Rasip1 is not only required for the cord-to-tube transition during early vasculogenesis, but it is also required for growing blood vessel lumen maintenance and remodeling during later embryogenesis. This requirement, however, does not last into adulthood, as Rasip1 is dispensable in quiescent, established blood vessels. Rasip1 is required, however, for angiogenic sprouting and

remodeling, in both growing retinal vessels and in a model of adult angiogenesis. Together, this work extends and confirms our original findings, but also suggests that Rasip1 may be a useful target for development of anti-angiogenic therapies.

## MATERIALS AND METHODS

### Animals

Conditional Rasip1 founders were generated by blastocyst injection of ES cells with targeted Rasip1 allele (ID:119405), obtained from KOMP EUCOMM. LacZ and Neomycin resistance cassette were flanked by Flp Recombinase Target (FRT) sites and removed by breeding with Flpe mice to generate Rasip1 floxed allele ($Rasip1^{f/f}$). $Rasip1^{f/f}$ mice were bred with Flk1-eGFP, Sox2-Cre, Tie2-Cre, Cdh5(PAC)-$Cre^{ERT2}$, CAG-$Cre^{ERT2}$, Rosa26-YFP, and Rosa26-Tomato [17–21]. Primers used to detect floxed, wildtype and mutant alleles were the following sequences: ATGGTATGCCTGCCATTTGT (sense), CGACGTCACTGTGTTCCACT and GGTCCTGTGAAAGAGCAAGC (anti-sense). Western blots were carried out with proteins extracted from mouse tissue using antibodies to Rasip1 (Novus Biologicals NB300-967) and β-actin (Cell signaling 3700).

### Histology of mouse embryo and postnatal tissue

Experiments were performed in accordance with protocols approved by UT Southwestern Medical Center IACUC. Mouse embryos and adult tissues were fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C. Postnatal retinas were fixed in 4%PFA/PBS for 2hr at room temperature as previously described [22].

### Analysis of angiogenesis in the postnatal mouse retina

To delete Rasip1 floxed site in the postnatal mice, tamoxifen (Invitrogen) (3mg of tamoxifen/40g mouse body weight) in corn oil (Sigma) was gavaged to mother mice six times from postnatal day 0 (P0) to P5. Eyes were collected at P6 and fixed in 4% PFA for 3 hr at room temperature. Retinas were dissected and immunostained as whole mount as previously described [23]. For Bromodeoxyuridine (BrdU) labeling, 300μg of BrdU (Invitrogen) was injected into P6 pups by intraperitoneal (IP) injection 2.5h before sacrifice. To visualize vascular lumens, 500μl of 15mg/ml FITC-Dextran (Invitrogen) was injected into left ventricle.

### Directed In vivo Angiogenesis Assay

Rasip1f/f; Cdh5(PAC)-CreERT2 mice were treated with tamoxifen (3mg of tamoxifen/40g mouse body weight) at 4 weeks old every other day for two weeks. At 8 weeks old, 20μl silicone cylinders (DIVAA angioreactors) containing basement membrane extract (BME), composed primarily of Laminin I, Collagen IV, and Entactin, with FGF-2 and VEGF (AMS Biotechnology, cat# 3450-048-SK) were embedded subcutaneously (bilaterally) in the dorsal flanks of mice and harvested 2 weeks later. Following the harvest of the angioreactor, the content (BME-blood vessel mixture) was digested according to the manufacture's instruction. The cells were collected by centrifugation, washed and further incubated with FITC-labeled *Griffonia Simplicifoli*a Lectin I at 4°C overnight. Endothelial cell abundance

in the defined volume of matrix was determined by staining cells with FITC-lectin and quantification by fluorometer (excitation 485nm, emission 510nm).

### In Situ Hybridization

Whole mount *in situ* hybridization was performed as previously described [7]. Embryos and retinas were fixed overnight at 4°C and incubated with DIG-labeled RNA probes overnight at 65°C. Tissues were washed with post-hybridization solutions, and then incubated in antibody overnight at 4°C. Color development was carried out using BM purple (Roche). Dig-RNA probes were generated from clones (Open Biosystems): CX40 (BC053054) and Dll4 (BC042497).

### Immunofluorescence - sections

Fixed tissues were washed with PBS. For paraffin sections, fixed tissues were dehydrated in series of ethanol washes, incubated in xylene, and then embedded in paraffin. For cryosections, fixed tissues were incubated in 30% sucrose/PBS at 4°C overnight, and then embedded in Tissue-Tek O.C.T. Embedded tissues were sectioned at 10μm by Microtome or Cryostat.

Sections were blocked for 30min. at room temperature in Cas-Block (Invitrogen). Primary antibodies in Cas-Block were incubated at 4°C overnight. Slides were then washed in PBS then incubated in secondary antibody for an hour at room temperature followed by incubation in DAPI. Immunoflorescence stained slides were mounted with ProLong Gold Antifade Reagent (Life Technologies).

Primary antibodies used to recognize the following proteins: PECAM (BD Biosciences 553370), Endomucin (Santa Cruz sc-65495), Rasip1 (Novus Biologicals NB300-967), VE-cadherin (Santa Cruz sc-6458), ZO-1 (Invitrogen 40-2200), Collagen IV (Millipore AB756P), pH3 (Millipore 06-570), Ki67 (Abcam ab15580), Cleaved Caspase 3 (Cell Signaling9661), GFP (Aves GFP-2010).

### Immunofluorescence - whole mount

Fixed embryos were incubated in 5% $H_2O_2$/ethanol at room temperature for 1 hour, and then rehydrated in series of Ethanol/PBS and 0.1% PBST washes. Embryos were blocked in Cas-Block for 2 hours at room temperature, then incubated in primary antibody diluted in Cas-Block at 4°C overnight. Embryos were washed in PBS, and incubated in Biotin conjugated Secondary antibody diluted in Cas-Block at 4°C overnight. Washed embryos in PBS were then incubated in ABC Elite reagent (Vector Lab), followed by Tyramide solution (Invitrogen TSA kit).

Fixed neonatal retinas and ear tissues were permeabilized in 1%Triton X-100 in PBS, and incubated in primary antibody in 1% Triton X-100 in PBS at 4°C overnight. Tissues were washed with PBS, and incubated in secondary antibody in 1% Triton X-100 in PBS at 4°C overnight. Immunostained retinas were cut and mounted in ProLong Gold Antifade reagent (Invitrogen).

### Imaging and quantification

Images were taken with Zeiss Axiovert microscope using DP70 camera (Olympus), LSM 710 Meta Zeiss Confocal microscope, or Zeiss Discovery Stereomicroscope. All data sets were taken from n≥3 biological replicates (embryos or retinas), with n≥5–10 fields of view analyzed. Data are presented as mean ± s.e.m. All statistical analysis was performed using two-tailed, unpaired, Student's t-test in Graphpad Prism software. P-values lower than 0.05 were considered statistically significant.

### Isolectin perfusion

E9.5 embryos were dissected, and yolk sacs were carefully torn for clear view of pumping hearts. 594-Isolectin (Invitrogen) was loaded into a fine needle and injected to left ventricle. After isolectin injection, its mobility through aorta was observed, and pictures were taken by DP70 camera (Olympus).

To visualize postnatal retinal perfusion, 0.0125g FITC-Dextran (Sigma) was dissolved in 1ml of 0.5% PFA/PBS was prepared. 0.5ml of the FITC-Dextran solution was injected into the left ventricle of an anesthetized P6 pup. After 5 min, retinas were dissected out and fixed in 4% PFA for 2 hours at room temperature. The retinas were rinsed with PBS, and then mounted on the slide with ProLong Gold Antifade reagent (Invitrogen).

### In vitro sprouting assays

Human umbilical vein endothelial cells (HUVECs) were utilized in 3D collagen matrix assays to induce tubulogenesis to mimic the processes of vasculogenesis or angiogenic sprouting using a defined serum-free system [24]. Four recombinant growth factors (obtained from R&D Systems) were added to 3D collagen matrices each at 200 ng/ml; fibroblast growth factor-2 (FGF-2), stem cell factor, interleukin-3 and stromal-derived factor-α. The culture media was Medium 199 with a 1:250 dilution of reduced serum supplement II (containing insulin) [8] and FGF-2 (40 ng/ml). ECs were treated with siRNAs directed to Rasip1, Arhgap29 (or both) versus control and then these assays were performed for 16 to 72 hr. EC tube formation was quantitated by tracing lumen areas using Metamorph software and EC tip cell numbers were quantitated by identifying elongated ECs with finger-like projections in light microscopic images. siRNAs from Ambion were: Control (AM4637) Silencer Select Negative Control #2;Rasip1 (s29763) 5′-CGAGCUGUUCAAAUCCGAA-3′, Arhgap29 (s485) 5′-GACCAAGGCUAAAACGAAU-3′.

### Permeability assay

0.5% Evans Blue (Invitrogen) solution in PBS was injected in the tail vein of the mice. To measure permeability at skin surface, 50ng of VEGF in 500μl of PBS or PBS alone were applied by intradermal injection. After 30 min, the mice were sacrificed and organs of interest were collected and weighed. Each tissue samples were incubated in 500μM formamide at 55°C overnight. Concentration of dissolved Evans Blue in formamide was measured by spectrophotometer (NanoDrop2000C, Thermoscientific) with absorbance at 610 nm.

# RESULTS

## Rasip1 is essential for initial blood vessel formation

We previously showed that Rasip1 global null (Rasip1$^{-/-}$) mouse embryos fail to form functional vascular tubes during initial blood vessel formation (or vasculogenesis), around embryonic day (E) 8.5 [7], resulting in 100% lethality. To test this requirement during angiogenesis and adult vessels, we generated conditional Rasip1 (Rasip1$^{f/f}$) mice, using ES cells from the Knockout Mouse Project Repository (KOMP). We then tested deletion of Rasip1 ubiquitously by crossing Rasip1$^{f/f}$ mice to Sox2-Cre, and conditionally in ECs using Tie2-Cre or Cdh5(PAC)-Cre$^{ERT2}$ (Fig. 1A).

In the presence of Cre, exon 3 of *Rasip1* is deleted in Rasip1$^{f/f}$ mice, resulting in a nonsense mutation (Fig. S1). During generation of the transgenic allele, a cassette containing the *LacZ* reporter and neomycin positive selection gene (LacZNeo) was inserted between Rasip1 exons 2 and 3, flanked by FRT site sequences. β-galactosidase staining of embryos containing this cassette showed EC specific expression at E8.5 (Fig. 1B–C). *Rasip1*$^{LacZNeo/+}$ and Rasip1$^{LacZNeo/LacZNeo}$ mice were bred to a *Flk1-eGFP* transgene to help visualize ECs. Rasip1$^{LacZNeo/LacZNeo}$ embryos displayed narrower dorsal aortae at E8.5, as seen in whole mount and cross sections of the embryos, suggesting that insertion of the LacZ and Neo genes may disrupt Rasip1 expression (Fig. 1D–G). Closer inspection of the vessels revealed failed lumen formation (Fig. S2) and an inability to polarize the tight junction marker ZO-1 away from the apical membrane (Fig. S2F). These data indicate that this mouse line recapitulates the global *Rasip1* knockout.

To completely delete Rasip1, the *LacZNeo* cassette was excised by crossing the mice to FLP recombinase transgenic mice, leaving behind the floxed allele (Rasip1$^{f/f}$). Rasip1$^{f/f}$ was then crossed to Sox2-Cre to delete Rasip1 ubiquitously throughout the epiblast. This resulted in embryos with vascular tubulogenesis failure identical to that seen in our previously generated Rasip1 global null mouse [7] (Fig. 1H–O). Antibody staining for Rasip1 showed complete loss of Rasip1 protein when using Sox2-Cre (Fig. 1H–K). Staining for VEcad revealed that ECs do not segregate the apical and lateral membranes properly, as adhesions are not cleared away from the apical membrane (Fig. 1L–O). *In situ* hybridization for the gene connexin 40 (*CX40)*, expression of which is dependent on normal blood flow [25], showed that Rasip1 is also necessary for arterial fate in the aortae, as previously shown (Fig. S3) [7].

To investigate the function of Rasip1 in the vasculature during vasculogenesis, Rasip1$^{f/f}$ mice were crossed to Tie2-Cre, which reportedly expresses Cre recombinase in angioblasts and in ECs [26]. We found that some, but not all Rasip1$^{f/f}$;Tie2-Cre embryos displayed tubulogenesis defects at E8.5 (Fig. 2). This was in contrast to the fully penetrant and global vascular failure seen in Rasip1$^{f/f}$;Sox2-Cre and Rasip1$^{-/-}$ mice, which always displayed occluded and discontinuous lumens at the onset of vasculogenesis. Generally, wild type (WT) E8.25–8.5 (5–8 somite stage) control embryos displayed patent dorsal aortae with continuous lumens (Fig. 2A,D). By contrast, Rasip1$^{f/f}$;Tie2-Cre embryos produced heterogeneous and mosaic lumen phenotypes in the dorsal aortae. About half of the embryos visually displayed occluded vessels (n=10) (Fig. 2B,E), while the other half had normal

dorsal aortae with open lumens (Fig. 2C,F). Of all of the embryos observed in cross section, about 40% of the aortae (n = 10) had failed lumen formation.

Of note, Tie2-Cre is also expressed in hematopoietic cells, however we did not observe defects in this cell type upon Rasip1 Tie2-Cre deletion. This is likely because Rasip1 is not expressed in early blood cells.

## Rasip1 deletion efficiency with different Cre lines

The variable observed phenotypes led us to examine efficiency of Rasip1 protein deletion in ECs using immunofluorescent staining. Cre expression, assessed with the Rosa26-YFP reporter, was clearly detected in early angioblasts at E8.0 (0–2 somite stage) (Fig. S4), suggesting gene deletion by Cre expression initiated in EC precursors. Antibody staining for Rasip1, however, unexpectedly revealed that Rasip1 protein was still present in PECAM and endomucin positive ECs (double stained, hereby designed as 'PE') in Rasip1$^{f/f}$;Tie2-Cre embryo at 5s (Fig. 2G–H,K–L). By 10s, however, Rasip1 protein was detectable in only 38% of embryonic ECs (Fig. 2I–J,M–O). In embryos with fully deleted Rasip1, failed tubulogenesis was observed in aortic ECs and tight junction molecules Claudin5 and ZO-1 and adherens junction molecule VEcad failed to clear from the apical membrane (Fig. S5A–L), suggesting polarity defects. However, the apical marker Podxl retained its normal localization at the apical membrane (Fig. S5M–R). Together, these findings suggest that the Tie2-Cre transgene only deletes Rasip1 prior to lumen opening in approximately 50% of Rasip1$^{f/f}$;Tie2-Cre embryos, preventing lumen formation during vasculogenesis.

## Rasip1 is required for embryonic vessel maintenance

Although some Rasip1$^{f/f}$;Tie2-Cre embryos did not exhibit vascular defects before E8.5, all embryos older than E8.75 displayed profound vascular defects. Anti-PECAM whole mount staining revealed that 100% of Rasip1$^{f/f}$;Tie2-Cre dorsal aortae at E8.75 had a constricted appearance and discontinuous lumens (Fig. 2P–Q). Discontinuous and constricted vascular lumens in aortae were shown in transverse sections through the embryonic trunk (Fig. S6C–D). In addition, arterial endothelial fate was affected as evidenced by loss of *CX40* gene expression in dorsal aortae of Rasip1$^{f/f}$;Tie2-Cre embryos (Fig. 2R–S). 100% of Rasip1$^{f/f}$;Tie2-Cre embryos displayed hypoplasia by E9.0 (Fig. S6B). To further determine functionality of blood vessels upon Rasip1 deletion with Tie2-Cre, we assessed blood circulation by injecting fluorescent Isolectin B4 (IB4) into the pumping heart of E9.5 mice. Injected IB4 in WT embryos rapidly traveled through the dorsal aortae and reached the caudal tail (Fig. 2T). By contrast, IB4 in the *Rasip1$^{f/f}$;Tie2-Cre* mutant embryos failed to circulate properly down the aortae and remained in the heart despite a normal heartbeat rhythm and strength (as per visual observation) (Fig. 2U). These results suggest that deletion of Rasip1 using Tie2-Cre blocks blood circulation in the developing embryo due to occluded vessels.

Furthermore, these results show that the vessel failure phenotype in Rasip1$^{f/f}$;Tie2-Cre embryos is distinct from Rasip1$^{f/f}$;Sox2-Cre or Rasip1$^{-/-}$ embryos. In Rasip1$^{f/f}$;Tie2-Cre embryos, residual Rasip1 protein remains in ECs due to inefficient Rasip1 deletion prior to lumen formation. This allows about half of the embryos to undergo normal tubulogenesis in

the dorsal aortae. 50% of the embryos formed vascular lumens at stages E8.0–8.5 (1–8s), but 100% of the embryos display occluded aortic lumens by E9.0. Together, these data suggest that embryonic vessels collapse after Rasip1 deletion, even if they initially open lumens. This indicates that Rasip1 is required to maintain vascular lumens during early development.

## Rasip1 is required for mid-gestation embryonic angiogenesis

Because all Rasip1$^{f/f}$;Tie2-Cre mutant embryos die by E10.5, Rasip1 function in later blood vessel lumen formation or maintenance cannot be assessed. To determine if Rasip1 is important for vascular development after early vasculogenesis, Rasip1 was deleted using the Cdh5(PAC)-Cre$^{ERT2}$ driver line [27]. This line expresses Cre recombinase under the control of the VE-cadherin promoter in an inducible manner. We first induced at E9.5 by tamoxifen gavage, and assayed vascular defects at E12.5 (Fig. 3). We found that Rasip1$^{f/f}$;Cdh5(PAC)-Cre$^{ERT2}$ embryos developed hemorrhages and died soon after vascular defects developed (Fig. 3A–B). We visualized vessels in the limb of Rasip1$^{f/f}$;Cdh5(PAC)-Cre$^{ERT2}$ embryos, using whole mount anti-PECAM staining, and found the limb vascular plexus disorganized with thin and disconnected vessels (Fig. 3C–F). Cross sections revealed that a number of vessels did not display open lumens (Fig. 3G–L), while others were swollen and enlarged (larger trunk vessels did not show obvious collapse). Quantification of vessel diameter revealed that 20% of capillaries failed to develop or maintain lumens (Fig. 3M). These results indicate that Rasip1 is required not only for the development and maintenance of vessels that form during vasculogenesis, but also for angiogenic expansion and remodeling that occurs within tissues at midgestation.

## Rasip1 is required for neonatal retinal vessel outgrowth

To study whether Rasip1 is required in blood vessels in other tissues and at other stages, we examined angiogenic sprouting during retinal vessel growth in postnatal mice. *In situ* hybridization of Rasip1 mRNA confirms restricted expression of Rasip1 in retinal blood vessel endothelium (Fig. S7A). Therefore, deletion of Rasip1 using the ubiquitous and inducible *CAG-Cre$^{ERT2}$* driver line should produce primarily EC autonomous defects after administering tamoxifen [18]. We carried out daily tamoxifen treatment of Rasip1$^{f/f}$;CAG-Cre$^{ERT2}$ or Rasip1$^{f/f}$ mice from postnatal day (P) 0 to 5, and retinas were analyzed at P6 (Fig. S7B). Vessels were visualized using isolectin B4 staining (Fig. S7D) and efficiency of Cre recombinase expression was examined using Rosa26-YFP (Fig. S7E). Following tamoxifen application, over 80% of retinal vessel ECs were YFP$^{+}$ (Fig. S7C–E), indicating significant deletion of Rasip1 was achieved in this vascular bed.

To assess the requirement of Rasip1 in angiogenesis, neonatal retinal vessels were examined for possible defects in vessel growth, remodeling, or lumen formation. Blood vessels in the retina grow strictly by angiogenesis, by way of sprouting and remodeling. Capillaries first enter the retina from the optic disc and then extend outward radially in a planar fashion, atop the retinal tissue that abuts the vitreous cavity [28].

Analysis of retinas following Rasip1 deletion showed significantly decreased blood vessel growth from the center of the retina compared to controls (Fig. 4A–C). Quantification of the distance between the optical disc and vascular tip showed a 23% reduction in vessel growth

(Fig. 4C). To determine whether reduced outgrowth in Rasip1 deficient vessels resulted from decreased proliferation of ECs, BrdU incorporation was carried out. The number of BrdU positive ECs in Rasip1 mutant retinal vessels was equivalent to the control retina (Fig. 4D–F), suggesting that Rasip1 deletion does not affect EC proliferation, consistent with our previous findings [7]. It is known that formation of filopodia at growing tip cells is required for angiogenic sprouting [29]. The number of filopodia and sprouts were not significantly affected by Rasip1 deletion (Fig. 4G–I). However, Rasip1 depletion led to increased vascular density within the capillary plexus (Fig. 4J–L). Vessel bed density (area) increased by 18.5% (P value =0.0025) (Fig. 4L). Close inspection of the vessels revealed that ECs were rounded with nuclei exhibiting decreased length (54% shorter) (Fig. 4M–O). Based on these results, we propose that vessel outgrowth is reduced in the absence of Rasip1, likely due to defects in EC morphology and failure of capillary plexus remodeling.

## Rasip1 deficiency causes blood vessel instability and angiogenesis defects

In addition to increased vascular density, Rasip1 deficient retinal vessels also displayed poorly organized capillary plexuses. To examine vessel stability in Rasip1 deficient plexuses, type IV collagen was examined to visualize EC tracks. We found that in regions of Rasip1 deletion, anti-collagen IV staining was observed to extend beyond IB4 positive blood vessels at the vascular front (Fig. 5A–C″). We speculated these areas to represent residual blood vessel basement tracks left by retracting tip vessels, suggesting failure in stabilization of remodeling vessels in the absence of Rasip1. Rasip1 deficient vessels also often displayed thin and elongated angiogenic sprouts at the periphery of the vascular front (Fig. 5D–F).

In WT vessels, two sprouts develop at the leading edge of the plexus and fuse by anastomosis to become part of the vascular plexus [30–32]. In Rasip1 deficient vessels, however, the sprouts appeared to lose the ability to fuse with neighboring sprouts. In addition, vascular stability was disrupted as hemorrhages and extravascular blood were observed (data not shown). This data suggests that Rasip1 is likely necessary for anastomosis and to restrain the length of angiogenic sprouts.

## Rasip1 is required for neonatal retinal vessel lumen formation

During vasculogenesis, Rasip1 was identified as a critical factor for EC tubulogenesis. To examine whether Rasip1 also controls lumen formation during angiogenesis, fluorescein isothiocyanate (FITC)-dextran was injected into the vasculature by perfusion. In WT retinas, dextran traveled throughout the capillaries and perfused the entire retinal vascular bed (Fig. 5G). This suggested that most retinal vessels are lumenized. By contrast, Rasip1 depleted vessels exhibited regions with no dextran at the leading edge of the vascular front (Fig. 5H). Quantification of the dextran perfused area in growing vessels showed a 72% reduction in the mutants (Fig. 5I). Although dextran did not reach the edge of the vascular bed, it was present at the center of the retina. Altogether, these data suggest that Rasip1 is required for proper lumen formation and vascular plexus remodeling.

## Rasip1 deletion in adult blood vessels

To examine whether Rasip1 is required in established adult mouse blood vessels, we deleted Rasip1 in 8 week old mice using the inducible Cdh5(PAC)-Cre$^{ERT2}$. We injected Tamoxifen

every other day for two weeks to effectively delete Rasip1. We then confirmed Rasip1 deletion by polymerase chain reaction (PCR) using genomic DNA to detect the deleted Exon 3 allele (Fig. S8A). To confirm the deletion of exon3 from Rasip1 transcripts, exon 2 and 3 loci were examined from cDNA generated from tamoxifen induced adult Rasip1$^{f/f}$;Cdh5(PAC)-Cre$^{ERT2}$ lungs. While exon2 was detected in Rasip1$^{f/f}$;Cdh5(PAC)-Cre$^{ERT2}$, exon 3 was significantly reduced (Fig. S8B). To confirm Rasip1 protein depletion, Western blot analysis was performed. As expected, Rasip1 level was significantly decreased in Rasip1 deficient lungs [11] (Fig. S8C). To visualize the Rasip1 deletion efficiency, Rasip1$^{f/f}$;Cdh5(PAC)-Cre$^{ERT2}$ mice were bred with *Rosa26-Tomato* reporter mice. After tamoxifen induction, most ECs examined in Rasip1$^{f/+}$;Cdh5(PAC)-Cre$^{ERT2}$ and Rasip1$^{f/f}$;Cdh5(PAC)-Cre$^{ERT2}$ mice expressed tomato fluorescence in the ear skin vasculature (Fig. S8D). In addition, immunofluorescent staining for Rasip1 in the endothelium lining the adult dorsal aorta, showed that Rasip1 was not detected in Rasip1$^{f/f}$;Cdh5(PAC)-Cre$^{ERT2}$ ECs (Fig. 6A–B).

## Rasip1 dispensable in established adult blood vessels

Rasip1$^{f/f}$;Cdh5(PAC)-Cre$^{ERT2}$ mice were grossly normal and healthy after tamoxifen treatment. To examine possible vascular defects, ear skin vessels were immunostained with anti-PECAM. Rasip1 depleted vasculature appeared normal and morphologically indistinguishable from control vessels (Fig. 6C–D). Previously, Rasip1 has been shown to be necessary for blood vessel permeability in ECs *in vitro* [14,15]. To test permeability in Rasip1 depleted quiescent vessels *in vivo* in adult mice, we injected Evans Blue dye to assess vessel “leakiness”. Evans Blue dye binds to albumin, a serum protein that is impermeable to the endothelium under non-stimulated conditions. Under stress conditions or growth, blood vessel ECs partially lose close adhesion contacts increasing blood vessel permeability to small proteins such as albumin [33]. Injection of VEGF stimulates EC permeability [34]. As expected, subcutaneous injection of VEGF into mice perfused with Evans Blue in the serum caused the blue dye to permeate into the surrounding injection site (Fig. 6E). Injection of PBS, by contrast, did not stimulate perfusion of Evans Blue at the injection site, confirming that VEGF increases vascular permeability and allows serum proteins to leak between ECs. Interestingly, although Rasip1 was previously shown to increase vascular permeability *in vitro*, quiescent Rasip1 mutant vessels did not show increased leakage in the lungs, liver, kidney, or skin, either when stimulated by VEGF injection and under non-stimulated conditions (Fig. 6F–G). This experiment indicates that Rasip1 may not measurably regulate vascular permeability in adult mouse vessels, *in vivo*.

## Rasip1 is required for angiogenesis in vivo and in vitro

We next confirmed that Rasip1 is required for lumen formation in cultured human ECs. We also assayed the role of Rasip1’s binding partner Arhgap29. As previously reported [7], after reduction of Rasip1 or Arhgap29 in HUVECs with siRNA, ECs assembled into cords but failed to develop lumens compared to controls (Fig. S9A–B,E). Reduction of both Rasip1 and Arhgap29 led to the same phenotype of EC cord structures that lacked lumens (Fig. S9D–E).

Similarly, loss of Rasip1 and Arhgap29 led to defects in angiogenic sprouting *in vitro*. We performed angiogenesis assays where HUVECs were plated atop VEGF-containing collagen, into which they sprouted. Interestingly, although lumen formation was reduced after reduction of Rasip1, Arhgap29, or both (Fig. 7A–E), the distance of cell invasion into the collagen was not affected (Fig 7F). This suggests that Rasip1 and Arhgap29 are not necessary for invasion into collagen. To determine whether Rasip1 or Arhgap29 affects tip cell number in the invading sprouts, we quantified the number of tip cells after 16 or 72 hour of culture by examining cross-sections. After treatment with control siRNA, around 25 tip cells at the end of angiogenic tips developed after 16 hours (Fig 7G). After 72 hours, only 5 or fewer tip cells were clearly discernible. By contrast, after Rasip1 or Arhgap29 reduction, the number of tip cells remained similar to controls at 16 hours, but after 72 hours the number of tip cells remained high and depleted less over time. Depletion of Rasip1 and Arhgap29 did not change the number of tip cells between 16 hours and 72 hours, however these were long and thin, similar to tip cells observed in retinal vessels following Rasip1 depletion. Together, this suggests that in addition to controlling vessel tubulogenesis, Rasip1 and Arhgap29 signaling pathways control tip cell behavior during angiogenic sprouting.

### Rasip1 is required for adult angiogenic vessel growth in vivo

To investigate whether Rasip1 is required for angiogenesis in adult mouse vessels, similar to neonatal retinal vessels, we examined vessel sprouting in tamoxifen treated Rasip1$^{f/f}$;Cdh5(PAC)-Cre$^{ERT2}$ and control mice, using Directed *In Vivo* Angiogenesis Assay (DIVAA) angioreactors (Fig. 7H). This assay consists of silicon tubes containing basement membrane extract mixed with fibroblast growth facter-2 (FGF-2) and VEGF, which are implanted subcutaneously and allow assessment of growing vessels, as previously shown [35–37]. In this assay, host blood vessels sprout into the implant, providing a means to measure angiogenesis *in vivo*.

After two weeks of vessel growth, angioreactors were removed from the mice and evaluated for changes in the degree of angiogenesis. ECs expressing Cre recombinase were visualized by using Rosa26-Tomato reporter mice. Vessel growth was observed in angioreactors (Fig. 7I–J), first by visualization of the tomato-labeled vessels (indicating Rasip1 deleted ECs), which showed that Rasip1 depletion decreased EC invasion into the angioreactors (Fig. 7I′,J′). In addition, the total number of ECs invading the angioreactors was found to be significantly reduced in Rasip1$^{f/f}$;Cdh5(PAC)-Cre$^{ERT2}$ mice compared to those in control mice, as determined by subsequent EC isolation and staining with FITC-lectin (Fig. 7K). These results indicate that Rasip1 is required for growth factor (FGF/VEGF) induced angiogenesis from adult vessels.

## DISCUSSION

In this study, we examined the role Rasip1 during vasculogenesis, angiogenesis, and maintenance of ECs *in vivo*, using a Rasip1 conditional knockout mouse model. In a previous study, we identified Rasip1 as a critical regulator of blood vessel tubulogenesis in the developing mouse embryo [7]. Here, we show that Rasip1 is essential for embryonic angiogenesis and vessel maintenance, after initial tubulogenesis. Tie2-Cre or inducible

Cdh5(PAC)-Cre deletion of Rasip1 in embryonic or perinatal vessels, after lumen formation, caused severe defects in angiogenesis, maintenance of EC lumens and vascular remodeling. Interestingly, we found that Rasip1 was dispensable for established blood vessels. Ablation of Rasip1 in ECs of adult mice did not lead to vascular defects. However, nascent vessels that form via sprouting or remodeling angiogenesis, require Rasip1 both in the postnatal retinal vasculature and in growing subdermal adult vessels (DIVAA). Together, our findings suggest that Rasip1 is essential for growing vessels, but not for quiescent blood vessel stability or function.

## Rasip1 is required for lumen formation

We previously showed that Rasip1 is critical for EC tubulogenesis during vasculogenesis [7]. This idea was challenged by Wilson *et al*., who proposed that Rasip1 is not required for *de novo* lumen formation, as patent lumens were observed in their Rasip1$^{-/-}$ mice [14]. Using a second, independently generated conditional ablation mouse model (KOMP), we confirm here our initial findings that Rasip1 is indeed essential to formation of continuous, functional EC tube formation during vasculogenesis.

Using a newly generated Rasip1 floxed allele, Sox2-Cre driven Rasip1 deletion phenocopied previously observed blood vessel tubulogenesis defects. Global and early Rasip1 deletion results in failure of endothelial cell-cell adhesions to organize peripherally and clear from the apical membrane. These junctional failures result in consequent inappropriate stitching of ECs together and prevention of continuous lumen formation (Fig. 8A). This phenotype was also observed in embryos that contain a LacZNeo cassette between the second and third exon, which interrupts the Rasip1 coding region.

These results support our previous findings that Rasip1 is essential for vascular tubulogenesis. A few studies have shown that Rasip1 is necessary to maintain the integrity of EC cell-cell adhesions *in vitro* [12,7,14]. In these studies, reduction of Rasip1 resulted in jagged and permeable adhesions. Rasip1 was found to control cell adhesions by mediating activated Rap1 and by inhibiting RhoA via the GAP Arhgap29. Previously, these signaling cascades have been shown to regulate actomyosin contractility to maintain stability of cell-cell adhesions and prevent them from becoming jagged and active [38]. It is likely that the function of Rasip1 identified in some of these studies, in organizing maturing focal adhesion junctions, may reflects its role in reorganizing adhesions within vascular cords. ECs need to rapidly clear cell adhesions from the apical domain of vascular cords, while maintaining lateral cell-cell junctions, thereby forming a single lumen that allows blood flow. Similar to Rasip1 control of adhesion organization via Rap1-RhoA signaling, Rasip1 is likely using these same mechanisms to organize adhesions between ECs in vascular cords to drive lumen formation between adhesions.

## Rasip1 is required for angiogenesis

Following vasculogenesis, blood vessel beds expand via angiogenesis. Angiogenesis in adults has been extensively studied because of the role it plays in many pathologic conditions such as cancer, diabetic blindness, age-related macular degeneration, and rheumatoid arthritis. From these studies, a wealth of mechanisms, signaling pathways and

different molecules have been revealed to regulate angiogenesis. While roles for Rasip1 in EC behavior were previously tested *in vitro* showing its requirement for EC coalescence, cell motility, and migration, we speculated that Rasip1 was likely required for vascular events inherent in angiogenesis [12].

To test for an angiogenic role for Rasip1, we used three different model systems: neonatal retinal vessel angiogenesis, adult subcutaneous vessel angiogenesis, and angiogenesis in 3D collagen matrix. In the first model, we showed that Rasip1 deficient retinal vessels displayed angiogenic defects, including significantly reduced vessel outgrowth and higher vascular density (Fig. 8B). Cells within the vascular plexus displayed a rounded cell morphology, characteristic of cells with high internal contractility. We propose that absence of Rasip1 leads to high RhoA activity, causing remodeling defects that lead to higher plexus density and reduced vessel outgrowth. Work by *Post et al. 2013* and *Xu et al. 2011* demonstrated that Rasip1 suppresses EC contractility by suppressing RhoA-actomyosin signaling. RhoA activates myosin to enhance actomyosin contractility of the actin cytoskeleton and therefore internally contract the cell. By inhibiting RhoA, and therefore myosin, Rasip1 eases the tension produced by internal actin contraction allowing the cells to relax and elongate. This mechanism likely allows vessels in the retina to extend and form a proper vascular plexus.

## Rasip1 ablation impairs angiogenic tip cells and lumens

Tip cells in the retina, however, displayed a different effect upon loss of Rasip1. Elongated sprouts were often found at the edges of Rasip1 depleted plexuses, appearing to fail to make connections with neighboring sprouts. A similar phenotype was previously reported in macrophage specific Notch1 deficient mice. In this study, it was shown that macrophages interact with Dll4 positive tip cells and activate Notch signaling to promote anastomosis [39]. Furthermore, reduction of Rasip1 or its binding partner Arhgap29 caused *in vitro* generated sprouting vessels to maintain excess numbers of tip cells. This phenotype is likely associated with Notch-Dll4 signaling as mutants lacking Notch maintain high numbers of tip cells [40]. The possibility that impaired Notch signaling could cause failure of tip cell-stalk cell maintenance and anastomosis in Rasip1 depleted retinas, resulting in elongated vessels, warrants further investigation.

While our understanding of lumen formation during angiogenesis is still primitive, we asked whether Rasip1 plays a role in tubulogenesis of sprouting vessels in the retina. Since Rasip1 was known to be required for EC tubulogenesis [7], we tested for the presence of functional vessels by injecting fluorescent labeled dextran into control and Rasip1 deficient vessels to visualize lumens and assess patency. Dextran was detected in both control and Rasip1 mutant mice, but retinal vessels from Rasip1 mutant mice displayed perturbed and discontinuous lumens around the retinal plexus periphery, where newly forming vessels are located, suggesting that Rasip1 is required for proper angiogenic lumen formation.

## Rasip1 is dispensable in adult blood vessel maintenance

The importance of vascular maintenance has received attention as clinical treatments for vascular diseases have failed due to an interruption in vessel integrity [41]. Disruptions in signaling pathways involved in vessel maintenance cause the breakdown of vessels and

impair vascular homeostasis. Signaling pathways such as the VEGF, Tie2, PDGF, and Notch are important for junction stabilization and vessel integrity. Interruption of these pathways results in vascular abnormalities and malformations, causing severe clinical conditions. As Rasip1 is essential for embryonic tubulogenesis and angiogenesis, we expected that Rasip1 deletion in the adult would cause severe vascular problems. However, endothelial specific deletion of Rasip1 caused no obvious abnormalities in the vasculature of adult mice. Rasip1 deficient mice appeared healthy and lived long term without obvious chronic health conditions, even though the mice demonstrated robust deletion of Rasip1 protein and mRNA and Cre-mediated recombination. Proliferation, morphological appearance, and vascular permeability were not affected by absence of Rasip1. These data suggest Rasip1 may be dispensable in established, quiescent vessels.

Previously, it was demonstrated that Rasip1 is required for maintenance of EC permeability in cultured ECs [15,14,16]. Rasip1 was shown to function as a Rap1 effector to suppress RhoA activity, and thereby inhibits formation and contraction of stress fibers attached to VE-cadherin based adherens junctions. Rasip1 depletion leads to discontinuous junctions that allow increased cell permeability. These studies utilized Electrical Cell Impedance Sensing (ECIS) to measure the passage of current across an endothelial layer *in vitro*. We show that at least *in vivo* no gross changes in vascular permeability were detected in adult vessels in Rasip1 deficient mice using an Evans Blue assay, which measures the passage of albumin through blood vessels. These results are in contrast to previous *in vitro* findings. More sensitive *in vivo* permeability assays may also be necessary to detect the passage of small molecules across the endothelium in blood vessels following Rasip1 deletion. Additionally, further investigation will be required to determine if Rasip1 influences vascular permeability in response to other permeability stimulators such as histamine or pathological conditions. Alternatively, Rasip1 may not regulate junction integrity in quiescent ECs, or do so in parallel with other factors. However, our observations suggest it may primarily regulate junction stability in actively growing vessels, during both vasculogenesis and angiogenesis, as seen in retinal sprouting and remodeling vessels and throughout development.

## CONCLUSIONS

In this study, Rasip1 is identified as a critical factor regulating tubulogenesis and overall blood vessel morphogenesis, during vasculogenesis and angiogenesis *in vivo*, using Rasip1 conditional gene ablation. Deletion of Rasip1 in embryonic vessels that form by vasculogenesis caused failure in endothelial tubulogenesis, as previously shown [7]. We also show that in embryonic vessels, Rasip1 is required for embryonic lumen maintenance, even after initiation of lumen formation. Rasip1 loss after lumen formation caused collapse of new vessels resulting in vessel instability. During angiogenesis, Rasip1 depletion caused significant abrogation of angiogenic expansion, due to failure of vessel remodeling. Rasip1 removal also affected angiogenesis in adult mice, as shown in DIVAA experiment. However, Rasip1 depletion in established adult vessels did not cause defects in vessel lumen maintenance. Together, these findings suggest that Rasip1 is critical for angiogenesis in the developing vasculature, but not for maintenance of stable blood vessels.

Anti-angiogenesis research has received clinical attention, however despite rapid progress many basic questions remain unanswered. Further study of Rasip1 as an essential factor for angiogenesis and tubulogenesis will provide a better understanding basic mechanisms underlying angiogenesis and lumen formation. Given that Rasip1 is only necessary during angiogenesis and is dispensable for established vasculature, targeting Rasip1 has potential as an anti-angiogenic agent.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## HIGHLIGHTS

- Rasip1 is required for embryonic and postnatal sprouting and remodeling angiogenesis.
- Embryonic subdermal and postnatal retinal vessels lacking Rasip1 display remodeling defects and vascular lumen diameter variability.
- Integrity and stability of quiescent adult vessels does not require Rasip1.
- Vascular permeability in adult mouse subdermal vessels does not require Rasip1.

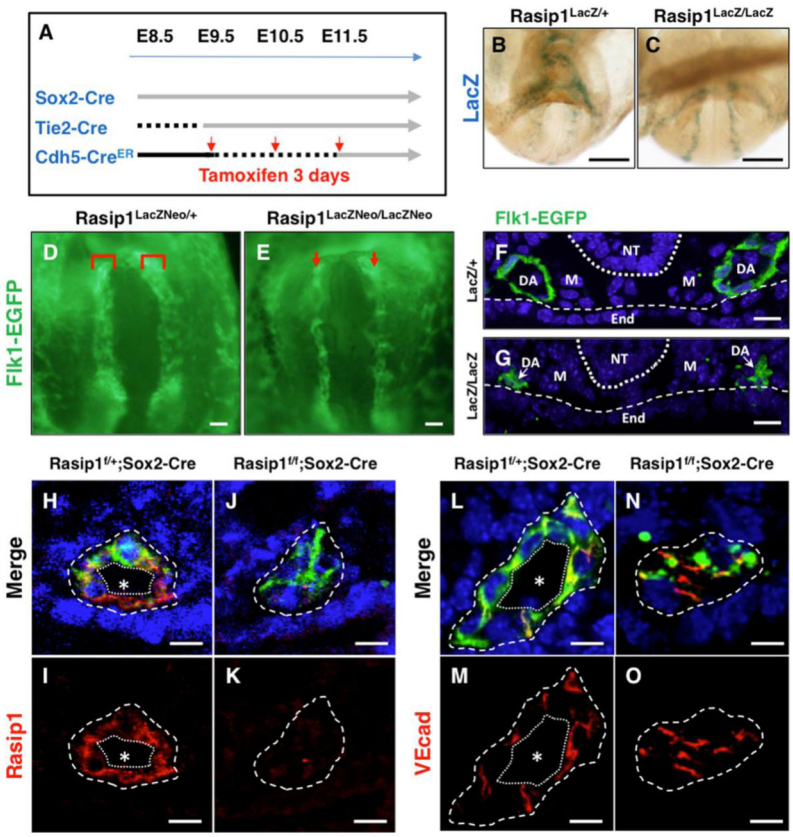


**Figure 1. Rasip1 is required for dorsal aorta lumen formation during vasculogenesis**

**(A)** A diagram denoting Cre expression and time of deletion with Sox2-Cre, Tie2-Cre, and Cdh5(PAC)-Cre$^{ERT2}$. Gray indicates deletion. **(B–C)** LacZ staining denoting the endothelial specificity of Rasip1$^{LacZNeo}$ mice in E8.0 embryos (1–3 somites). **(D–E)** LacZNeo genes disrupt function of the Rasip1 gene causing failure of dorsal aorta lumen formation. Flk1-eGFP transgene is expressed in the vasculature. Brackets mark open vessels and arrows mark the closed vessels. **(F–G)** Cross sections of Rasip1$^{LacZNeo}$;Flk1-eGFP mice. NT = neural tube, M = mesoderm, End = endoderm, DA = dorsal aorta. **(H–K)** Rasip1 (red), PECAM (green), and Endomucin (green) antibody staining with DAPI (blue) showing Rasip1 deletion using Sox2-Cre. Asterisks mark the vessel lumen. **(L–O)** Immunostaining for PECAM (green), Endomucin (green), VEcad (red), and DAPI (blue) showing Rasip1 deletion by Sox2-Cre prevents the segregation of adhesions away from the apical membrane. Scale bars: B–C 400μm, D–E 50μm, F–G 500μm, H–O 15μm.

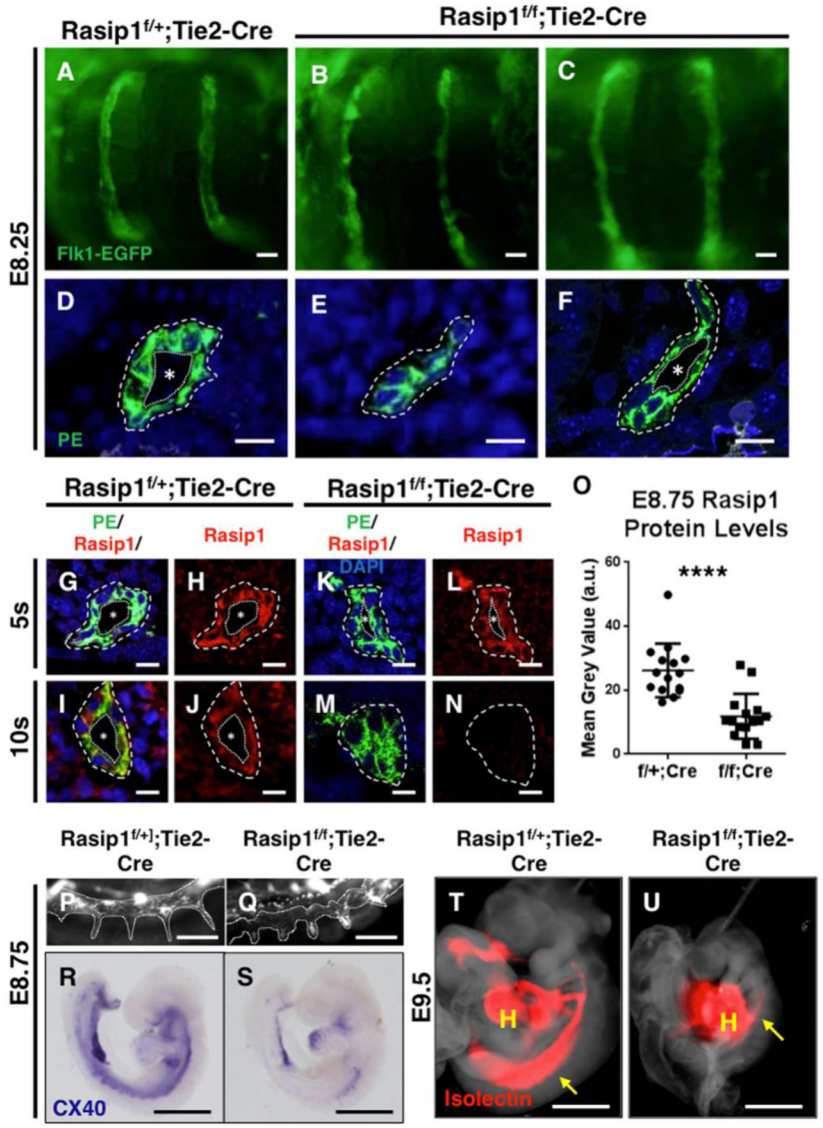


**Figure 2. Rasip1 is required to maintain dorsal aorta lumens and allow circulation**

**(A–C)** Rasip1 deletion using Tie2-Cre leads to failed lumen formation in the dorsal aortae of 50% of E8.25 embryos (n=10). **(D–F)** Cross sections through the dorsal aortae of E8.25 embryos. Green, Anti-PECAM and anti-Endomucin double immunofluorescence (PE). **(G–N)** Rasip1 protein is deleted by E8.75 (10s), but is not usually deleted during lumen formation stages (≤5s). Red, Anti-Rasip1 immunofluorescence. **(O)** Quantification of Rasip1 fluorescent intensity after deletion with Tie2-Cre at E8.75. **** = $p<0.0001$ n = 6. **(P–Q)** Wholemount PECAM staining shows that lumens collapse at E8.75 after Rasip1 deletion using Tie2-Cre. **(R–S)** *In situ* hybridization of the artery marker CX40 showing failed artery specification after deletion of Rasip1 using Tie2-Cre at E8.75. **(T–U)** Injection of fluorescent isolectin B4 into the heart reveals that circulation fails after deletion of Rasip1 using Tie2-Cre. H = heart. Yellow arrows show the extent of isolectin B4 circulation. Scale bars: A–C 50μm, D–N 15μm, P–G 200μm, R–U 1mm.

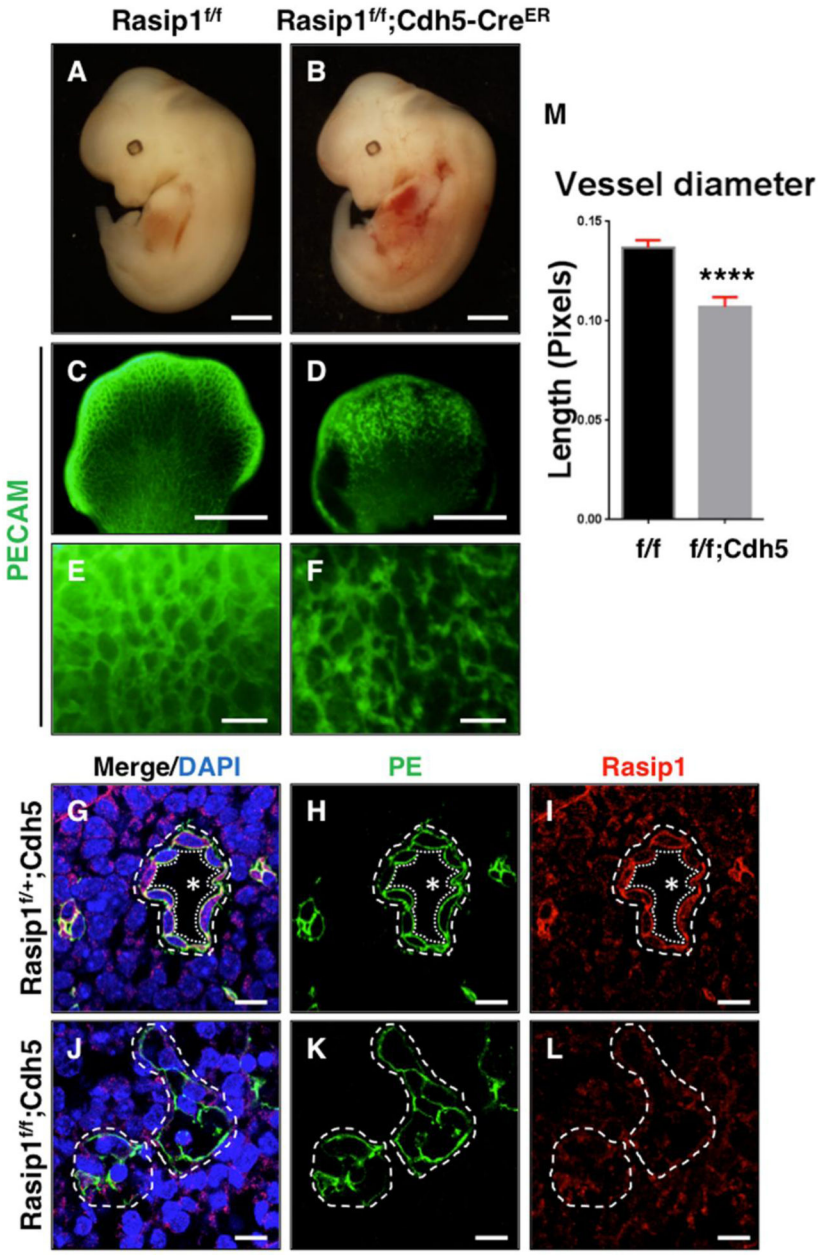


**Figure 3. Rasip1 is required for blood vessel maintenance and later embryonic vascular lumen formation**

**(A–B)** Rasip1 deletion at E12.5 using Cdh5(PAC)-Cre$^{ERT2}$ (or *Rasip1$^{f/f}$;Cdh5(PAC)*-Cre$^{ERT2}$) causes embryonic lethality and hemorrhaging. **(C–F)** Limb bud of Control and Rasip1 deleted vessels stained with PECAM in whole mount reveal constricted vessels. **(G–L)** Sections of Rasip1 deleted vessels in the limb bud stained for PECAM (green), Endomucin (green), and Rasip1 (red) show occluded blood vessels. Asterisks denote open vessels. **(M)** Quantification of blood vessel diameter in control and Rasip1 deleted vessels. **** = p<0.0001 f/+;Cre$^{ERT2}$ n = 122, f/f;Cre$^{ERT2}$ n = 137. Scale bars: A–B 3mm, C–D 1mm, E–F 25μm, G–L 30μm.

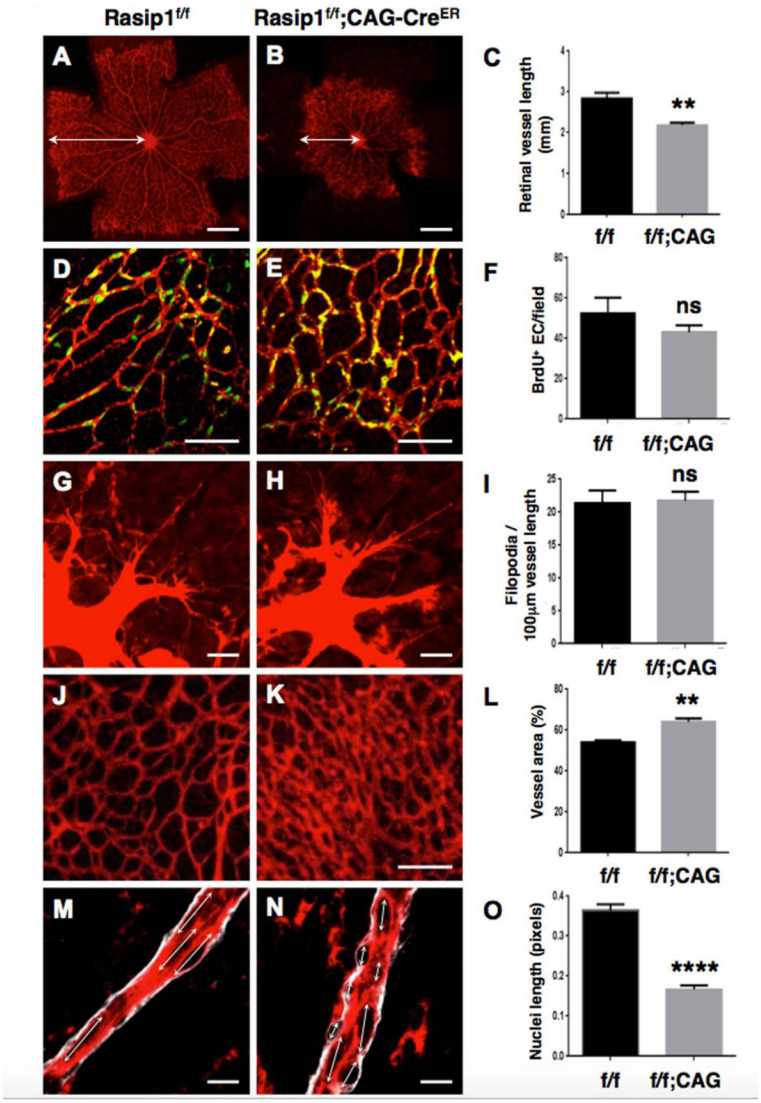


**Figure 4. Rasip1 is required for retinal vessel angiogenesis**

**(A–C)** Isolectin B4 staining (red) of control and Rasip1 deleted retinas (*Rasip1$^{f/f}$;CAG-Cre$^{ERT2}$*, or *Rasip1$^{f/f}$;CAG*) shows reduced vessel outgrowth; arrows mark the radius of the vessel; quantified in C. ** = p<0.01. **(D–F)** BrdU (green) injection into control and Rasip1 deleted pups show that Rasip1 does not affect the rate of EC proliferation; quantified in F. ns = not significant **(G–I)** Isolectin B4 staining shows that filopodia numbers are not affected in control or Rasip1 deleted retinas; quantified in I. ns = not significant. **(J–L)** Rasip1 affects vascular plexus density; quantified in L. ** = p<0.01. **(M–O)** ECs become less elongated after deletion of Rasip1; red = isolectin B4, white = collagen, quantified in O. **** = p<0.0001 n = 3. Scale bars: A–B 500µm, D–E 100µm, G–H 10µm, J–K 100µm, M–N 10µm.

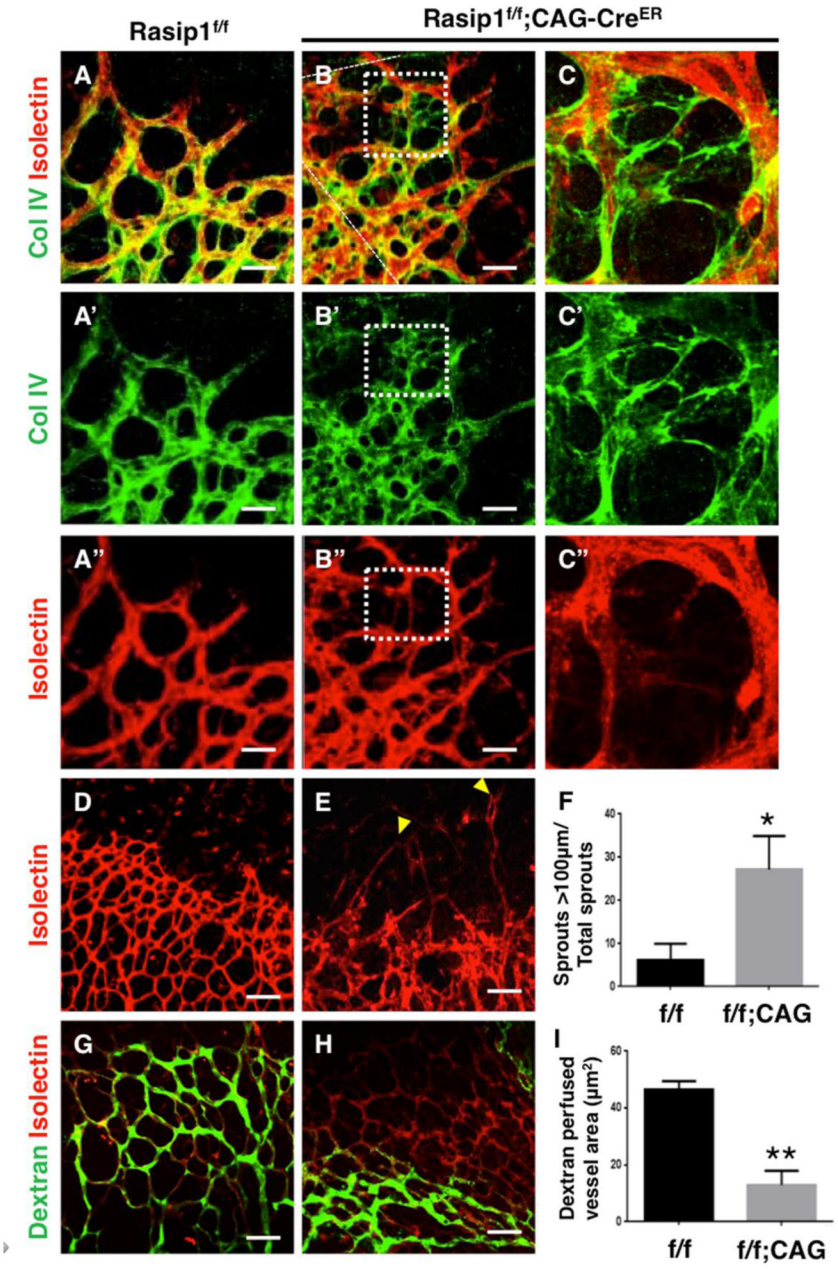


**Figure 5. Rasip1 is required for blood vessel stability**

**(A–C″)** Wholemount immunofluorescence for Isolectin B4 and Col IV. Col IV positive areas without blood vessels were detected in *Rasip1$^{f/f}$;CAG-Cre$^{ERT2}$* retinas indicating that vessel regression is increased in the absence of Rasip1. **(D–E)** Elongated sprouts (yellow arrowheads) were seen in *Rasip1$^{f/f}$;CAG-Cre$^{ERT2}$* retinas. **(F)** The number of sprouts longer than 100µm were quantified. ** = p<0.05 n = 3. **(G–H)** FITC-dextran was perfused into retinal vessel lumens. **(I)** Quantification of dextran perfused area shows that the luminal space is decreased in the absence of Rasip1. **p<0.01 n = 3. Scale bars: A–B″ 250µm, D–H 100µm.

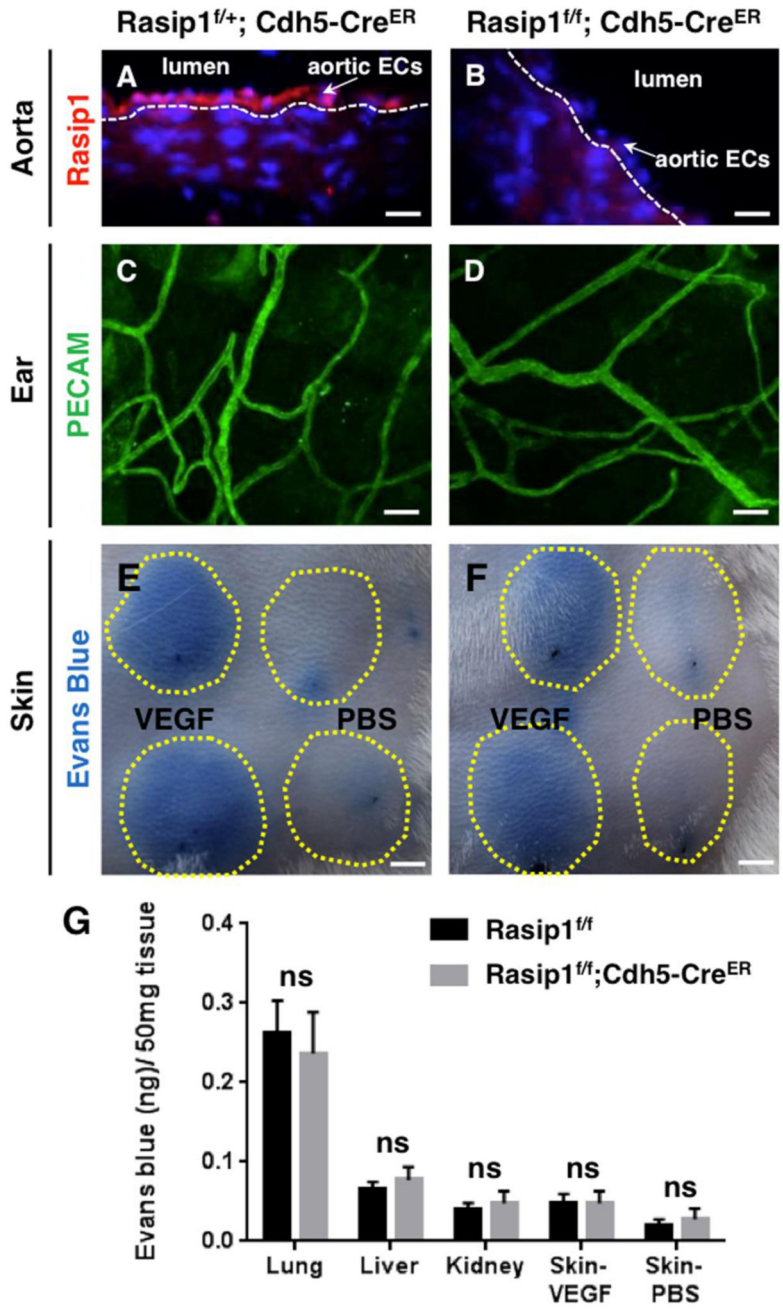


**Figure 6. Rasip1 is dispensable for adult vessel maintenance**

**(A–B)** Rasip1 staining in the aorta in control mice and after deletion using Cdh5(PAC)-Cre$^{ERT2}$. **(C–D)** PECAM staining in the ear vasculature shows no obvious defects after reduction of Rasip1 in adult mice. **(E–F)** Blood vessel permeability assay using Evans Blue dye. Mice perfused with Evans Blue were injected with VEGF or PBS. No differences were found in Evans Blue diffusion in control or Rasip1 depleted adult mice. **(G)** Quantification shows no difference in Evans Blue diffusion into lung, liver, kidney, or skin in control or Rasip1 depleted adult mice. Scale bars: A–B 50μm, C–D 25μm, E–F 5mm.

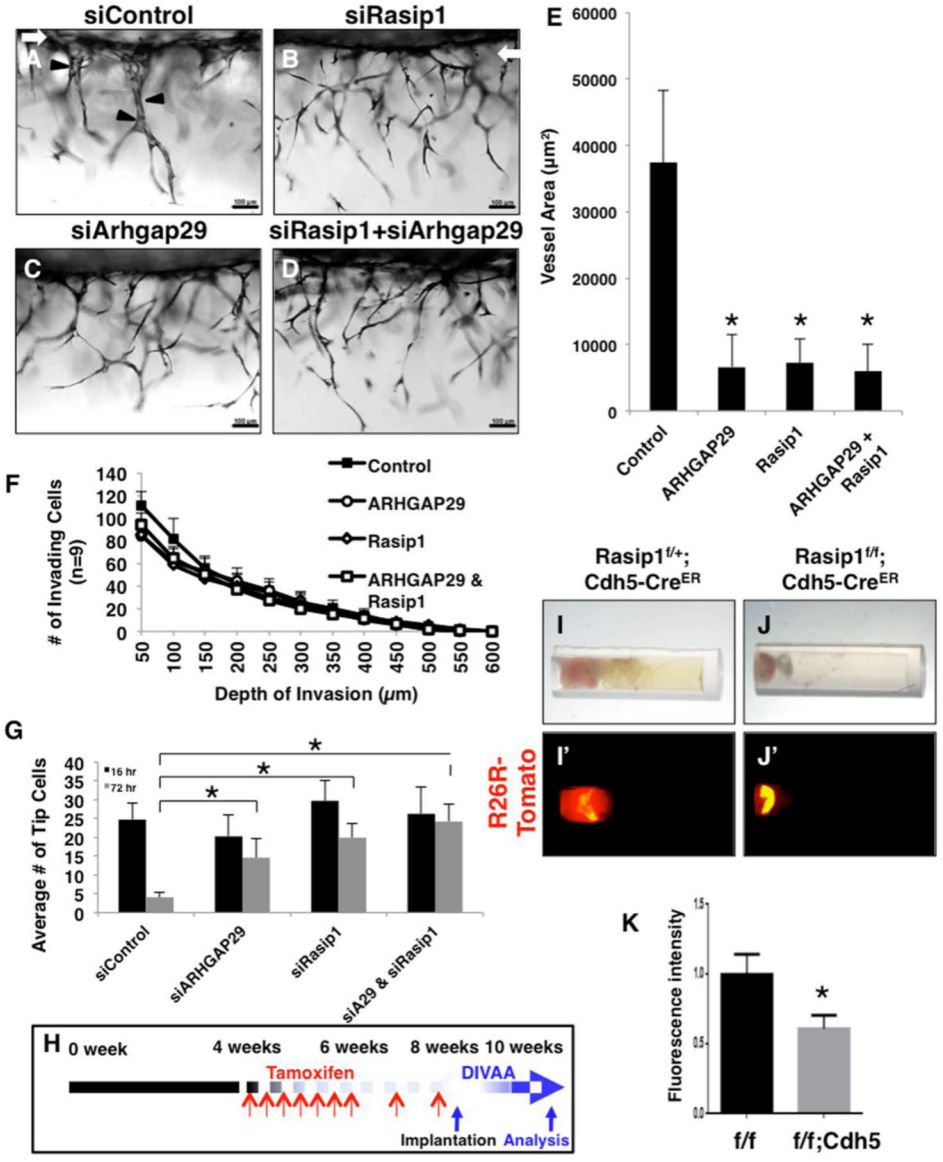


**Figure 7. Rasip1 is required for angiogenesis in human ECs and in adult subcutaneous vasculature**

(A–D) Angiogenesis assay using HUVECs plated onto collagen. Rasip1, Arhgap29, or both were depleted using siRNA. Arrowheads show open lumens. (E) Quantification of lumen formation in the angiogenesis assay. Reduction of Rasip1, Arhgap29, or both prevented lumen formation. * = $p<0.01$, n=15. (F) Quantification showing reduction of Rasip1, Arhgap29, or both together did not affect the extant of invasion in the angiogenesis assay. * = $p<0.05$. (G) Quantification of tip cell number after reduction of Rasip1, Arhgap29, or both at 16 and 72 hours of culture in the angiogenesis assay. Tip cell numbers did not change till 72 hours when more tip cells persisted after Rasip1and/or Arhgap29 reduction. * = $p<0.01$, n=15. Scale bars: E–H 100μm. (H) Schematic diagram of Tamoxifen induction and DIVAA experiment. (I–J) ECs invaded into the DIVAA angioreactor. (I′–J′) Rosa26-Tomato

reporter allele expression shows ECs in the angioreactor. (K) The number of invading ECs is measured by fluorescent intensity and normalized to the control. * =P<0.05, n=15.

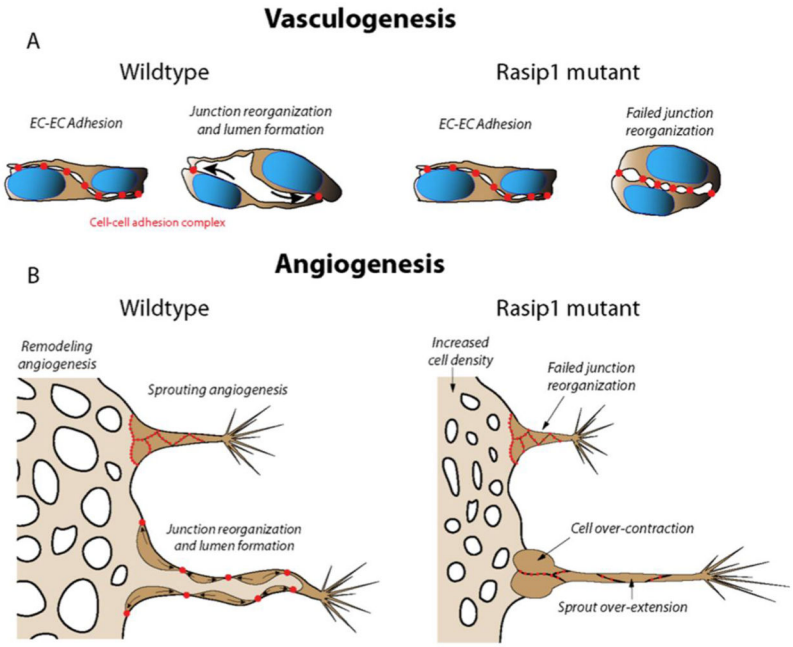


**Figure 8. Rasip1 regulates adhesion polarity and cell shape to control lumen formation and blood vessel morphogenesis**

Model of Rasip1 regulation of blood vessel morphogenesis during vasculogenesis (top) and angiogenesis (bottom). **(A)** Rasip1 regulates blood vessel lumen formation by controlling organization of endothelial cell-cell adhesions (red). Normally, cell-cell adhesions are cleared from the apical membrane to form a single open lumen. In the absence of Rasip1, vascular cords fail to segregate their adhesions peripherally, away from the apical membrane. **(B)** Rasip1 is also required for blood vessel angiogenesis. We demonstrate this in the retinal vasculature, where Rasip1 is required for both remodeling of the primitive vascular plexus and sprouting of nascent vessels. In these vessels, Rasip1 controls junction localization, and is also required for proper cell contractility in sprouting and non-sprouting ECs. Non-sprouting ECs become rounder in shape and sprouting ECs become unstable and elongate in the absence of Rasip1.